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Advances in Brief

Increased Copy Number at 20q13 in Breast Cancer: Defining the Critical Region and Exclusion of Candidate Genes¹

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Abstract

Studies by comparative genomic hybridization have indicated that a major new locus for DNA amplification in breast cancer is 20q13 and suggested that this genetic event is associated with aggressive clinical behavior. We used interphase fluorescence *in situ* hybridization with anonymous cosmid probes and gene-specific P1 clones to determine the minimal common region of increased copy number and to study involvement of known genes at 20q13. Based on high-level copy number increases (3 to 10-fold) found with one or more probes in 5 of 14 (35%) breast cancer cell lines and in 3 of 36 (8%) primary tumors, the critical region was narrowed to ~1.5 megabases at 20q13.2 defined by fractional length pter values 0.81–0.84. Previously known genes were excluded as candidates, implying that this chromosomal region harbors a novel oncogene that contributes to the malignant progression of breast cancer.

Introduction

DNA amplification is an important mechanism of tumor progression that allows cancer cells to up-regulate expression of critical genes such as oncogenes and genes conferring drug resistance (1–3). Three major loci of amplification found in 10–25% of primary breast carcinomas include the *ERBB2* oncogene at 17q12, *Cyclin-D* at 11q13, and *MYC* at 8q24 (4). Fibroblast growth factor receptor genes (*FLG* at 8p12 and *BEK* at 10q24) and several other genes at 15q24–q25 (*FES/IGFR-1*) have also been reported to be amplified (5, 6). Our recent studies by CGH⁵ indicate that besides the aforementioned genes and loci, almost 20 other chromosomal regions, most of them not associated with known oncogenes, are present at an increased copy number in breast cancer (7).

Increased copy number at 20q13 emerged as one of the most common genetic aberrations in breast cancer by CGH and was found in 18% of primary tumors and 40% of cell lines (7). Subsequently, copy number increase at 20q13 was shown to be strongly associated with poor prognosis in axillary node-negative breast cancer, suggesting that this genetic event confers a more aggressive tumor phenotype.⁶ The 20q13 region contains several genes that could contribute

to breast cancer progression (8, 9). These include, *e.g.*, topoisomerase I (*Topo I*), protein tyrosine phosphatase (*PTPNI*), *SRC* oncogene, adenosine deaminase (*ADA*), guanine nucleotide-binding protein (*GNAS1*), and zinc finger protein 8 (*ZNF8*).

In this study we sought to map this putative new oncogene locus in more detail by defining the minimal region of increased copy number in breast cancer cell lines and primary tumors by using FISH and a series of precisely mapped probes distributed along chromosome 20q. We report definition of a 1.5-Mb region of highly increased copy number at 20q13.2, exclusion of candidate genes, and suggest that a novel oncogene in this region is responsible for the more aggressive phenotype of certain breast cancers.

Materials and Methods

Probes. Twenty-four cosmid probes localized to 20q13 by metaphase FISH mapping and digital image quantitation of the fractional length from pter (FLpter) (10, 11) were used to assess copy numbers at multiple sites in 20q13. Closely spaced cosmids that could not be resolved by FLpter measurements were ordered by pair-wise two-color FISH to metaphase chromosomes and interphase nuclei (12). RMC20C038 (FLpter 0.237) was used as a reference probe in interphase FISH studies of tumor samples. In addition, most specimens were also tested with reference probes RMC20C038 (FLpter 0.121) and RMC20C041 (FLpter 0.593). P1 clones for known genes in the 20q13 region were obtained by screening a human genomic P1 library (DuPont, Boston, MA) using polymerase chain reaction with primers specific for these genes (10). Test probes were labeled with biotin-14-dATP and reference probes with digoxigenin-11-dUTP, using nick translation.

Preparation of Cell Lines and Primary Breast Tumors for FISH. Fourteen established breast cancer cell lines (BT-474, BT-549, DU4475, MCF-7, MDA-134, MDA-157, MDA-330, MDA-361, MDA-415, MDA-436, SK-BR-3, ZR-75-1, UACC-812, and UACC-893) as well as a nontumorigenic transformed breast epithelial cell line HBL-100 (all obtained from the American Type Culture Collection, Rockville, MD) were grown in recommended culture conditions. The cells were trypsinized after being kept at confluence for 5–7 days to obtain G₁-enriched interphase nuclei. The cell suspensions were incubated in 75 mM KCl for 15 min at 37°C followed by fixation in methanol-acetic acid (3:1). Fixed cell suspensions were dropped on slides. Similarly prepared foreskin fibroblasts were used for interphase FISH mapping and as negative controls in copy number increase studies. Normal peripheral blood lymphocyte metaphase chromosomes prepared by standard protocols were used for pair-wise two-color FISH.

Thirty-six primary breast carcinomas diagnosed at the Tampere University or City Hospitals during 1987–1991 were used in this study. Fifteen were node-negative and 21 were node positive. One tumor was a lobular invasive carcinoma, whereas the rest were ductal invasive carcinomas. Distribution of the tumors by grade (WHO grading system) was grade I (8 cases), grade II (18 cases), and grade III (10 cases). After histological verification that each tumor was representative and contained a high proportion of tumor cells, nuclei were isolated from 100 μ m frozen sections according to a modified Vindelov procedure for DNA flow cytometry and prepared for FISH as described (13).

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⁵ The abbreviations used are: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; Mb, megabase; FL, fractional length.

⁶ J. J. Isola and F. M. Waldman, manuscript in preparation.

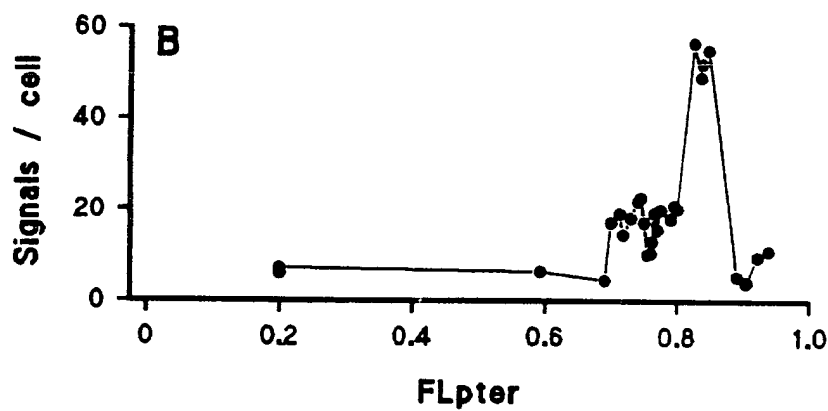
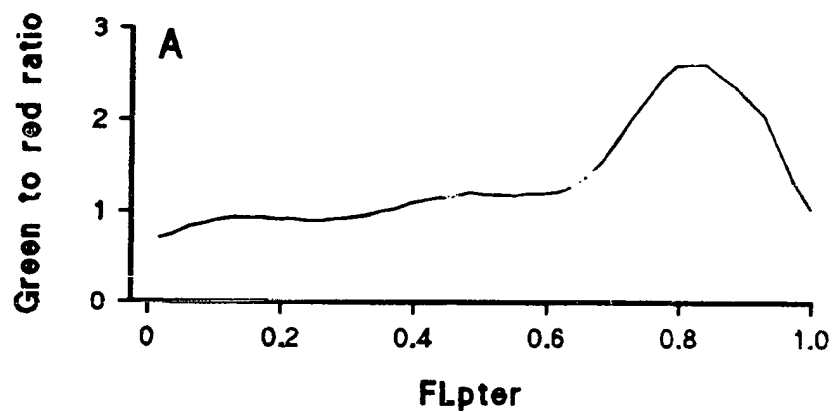


Fig. 1. Increased copy number at the 20q13 region in the BT-474 breast cancer cell line. A, according to CGH analysis (7), the BT-474 cell line shows an elevated green to red (tumor to normal) fluorescence ratio at 20q13 indicating that DNA sequences from this locus are present at an elevated copy number. B, distribution of cosmid and P1 probe signal copy number per cell by interphase FISH analysis as a function of the FLpter values for the probes indicates that the region of copy number increase in this cell line is defined by probes mapping to FLpter 0.80–0.86. C, a digital image of BT-474 cell nuclei hybridized with the minimal region probe (RMC20C001).



C



Fluorescence *In Situ* Hybridization. Two-color FISH was performed by using a biotin-labeled 20q13-specific probe essentially as described (14). The primary tumor samples were postfixed in 4% paraformaldehyde/phosphate-buffered saline for 5 min at 4°C prior to hybridization. Primary tumor slides were dehydrated in 70, 85, and 100% ethanol, air dried, and incubated for 30 min at 80°C. Cell preparations were denatured in a 70% formamide/2 × standard saline citrate solution at 70°C for 3 min, followed by proteinase K digestion (1 µg/ml). The hybridization mixture contained 18 ng of each of the labeled probes and 10 µg human placental DNA. After hybridization the probes were detected immunochemically with avidin-fluorescein isothiocyanate and anti-digoxigenin rhodamine. Slides were counterstained with 0.2 µM 4,6-diamidino-2-phenylindole in an antifade solution.

Fluorescence Microscopy and Interphase Scoring. A Nikon SA fluorescence microscope equipped with double band-pass filters (Chromatechnology, Brattleboro, VT) and a ×63 objective (NA 1.3) was used for simultaneous visualization of fluorescein isothiocyanate and rhodamine signals. At least 50 nonoverlapping nuclei with intact morphology based on 4,6-diamidino-2-phenylindole counterstaining were scored to determine the number of hybridization signals for the 20q13 test probe and reference probe. Leukocytes infiltrating the tumor were excluded from analysis. The scoring results were expressed both as the absolute hybridization signals per cell (absolute copy number) and as the number of signals relative to the reference probe (relative copy number). Samples with a relative copy number >3 were considered to have high-level copy number increase. control hybridizations to normal metaphase chromosomes and fibroblast interphase nuclei were done to ascertain that the probes recognized a single copy target and that the hybridization efficiencies of the test and reference probes were similar.

Results

DNA Sequence Copy Number Changes at 20q13 in Breast Cancer Cell Lines. FISH with carefully mapped and ordered probes for 20q13 (10) was more precise than CGH in defining the region and the level of copy number increase (Fig. 1). Interphase FISH studies showed high level increases in copy number (3- to 10-fold; 15–60 copies/cell) with one or more of the 20q13-specific probes in 5 of 14 breast cancer cell lines (Fig. 2). The region with the highest copy number in four of these cell lines was defined by a single cosmid, RMC20C001, with an FLpter value of 0.825. This same region also showed low-level copy number increase (1.5 to 3-fold; 4.5–6.5 copies/cell) in three other cell lines (MDA-361, MDA-134, and UACC-893).

MCF-7 breast cancer cell line showed a separate region of high-level copy number increase defined by four cosmids with FLpter values ranging from 0.74 to 0.76. UACC-812 cell line showed increased copy number both at RMC20C001 and at another region defined by two cosmids with a FLpter value of 0.78.

Analysis of Primary Breast Cancers. Three- to 6-fold relative copy number increase (19–23 copies/cell) was found in 3 of 36 unselected primary tumors (8%) by FISH with the RMC20C001 probe. In addition, 6 tumors (17%) showed slightly elevated copy number (1.5- to 3-fold, 6–8 copies/cell). The three tumors with high-level copy number increase at RMC20C001 were extensively studied with flanking probes (Fig. 2). These studies confirmed that RMC20C001 defines the region of highest copy number also in primary tumors.

Exclusion of Candidate Genes. According to recent physical mapping studies by Weissenbach (9) and ourselves (10), the following genes can be excluded as candidates based on their distance from the probe RMC20C001 (FLpter 0.825) that defines the critical region (FLpter): *HCK* (0.53), *GNRH* (0.53), *RPN2* (0.57), *SRC* oncogene (0.60), *Topo I* (0.67), *ADA* (0.68), *SEMG1* (0.68), *CHRNA4* (0.88), *GNAS* (0.88), *EDN2* (0.89), and *ZNF8* (0.93). The absence of high-level copy number increases of these genes was confirmed by FISH to interphase nuclei of the BT474 cell line using gene-specific P1 clones as probes. Protein tyrosine phosphatase (*PTPN1*, FLpter 0.78), melanocortin-3 receptor (*MC3R*, previously known as *D20S32E*, 0.81), and phosphoenolpyruvate carboxy kinase (*PCK1*, 0.85) were so close to the critical region that the entire panel of 20q13-positive cell lines and primaries was studied by interphase FISH using P1 clones to these genes. In most of the specimens evaluated, the region defined by the RMC20C001 probe was present at a higher copy number than any of these three genes (Fig. 3). This finding suggests exclusion of these genes as targets for the 20q13 amplification.

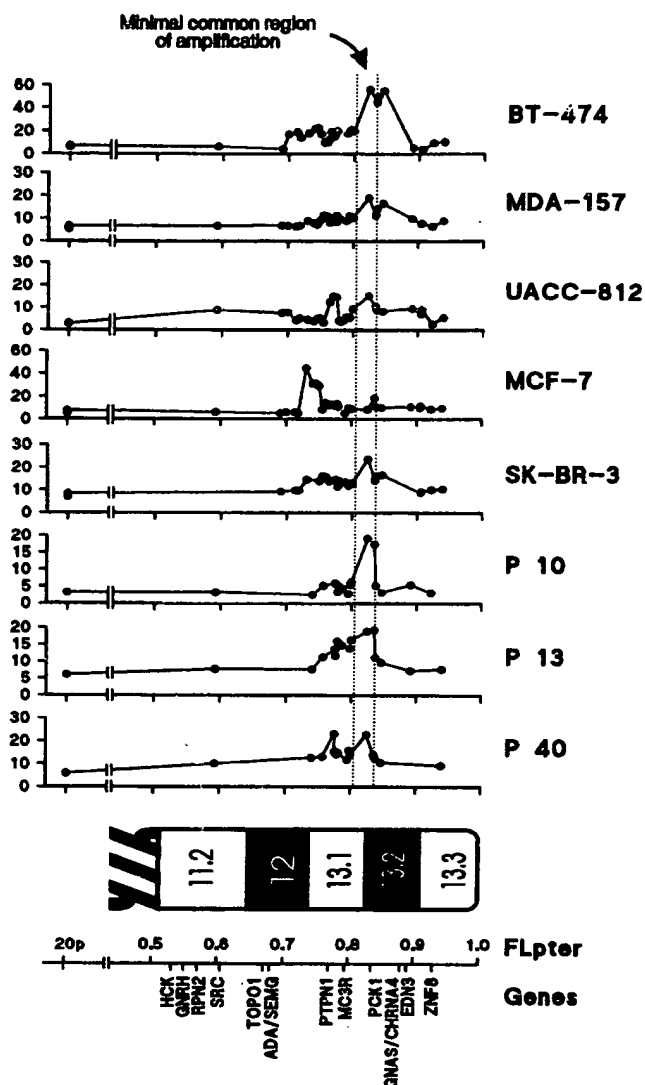


Fig. 2. Defining the minimal common region of high-level copy number increase in five breast cancer cell lines and three primary tumors (P10, P13, and P40), using P1 probes to known genes and physically mapped cosmid probes for 20q. The mean probe signal number per cell is shown as a function of the FLpter value. The minimal highly increased region involved in all cases, except in the MCF-7 cell line, is shown with dotted lines. The locations of known genes along 20q are shown at the bottom of the figure. The NIGMS ideogram of chromosome 20 is shown for approximate visual comparison.

Discussion

This study illustrates the potential of molecular cytogenetic techniques in the rapid identification and detailed mapping of genomic sites harboring putative oncogenes. The initial finding of increased copy number at 20q13 in breast cancer was based on the genome-wide copy number analysis using CGH (7). In this study, a narrow region highly increased in copy number was defined by FISH analysis of interphase nuclei from several breast cancer cell lines and primary tumors using a physically mapped and ordered set of large-insert genomic probes. The average distance between probes in the region was ~0.5 Mb. Analysis of regions of highest copy number in different primary tumors and cell lines indicated an ~1.5 Mb region at 20q13.2 as the site of the putative new oncogene. This corresponds to an at least 10-fold increase in mapping precision as compared to CGH data (7).

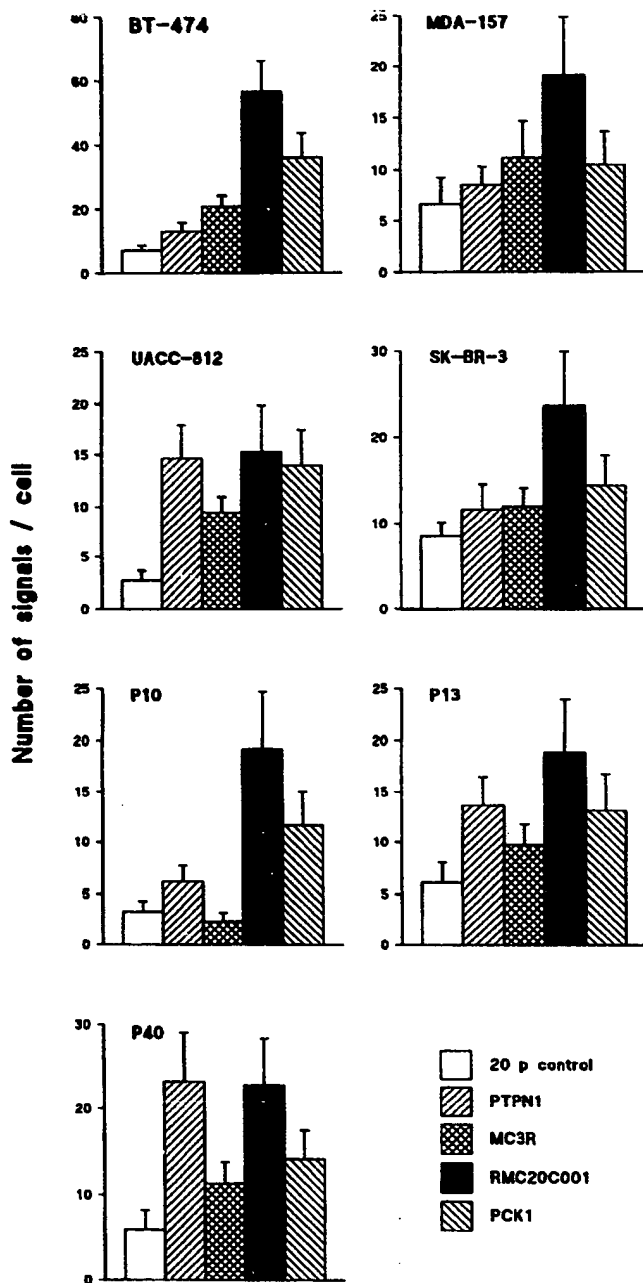


Fig. 3. Exclusion of candidate genes at 20q13. The mean copy number (\pm SD) obtained by interphase FISH with the gene-specific probes (PTPN1, MC3R, and PCK1) is compared with signal counts obtained with the RMC20C001 cosmid in four cell lines and three primary tumors.

The ability of FISH to quantitatively distinguish between the different levels of copy number increase at 20q13 was essential in narrowing down the common overlapping region of highest copy number. Detailed analyses of the amplicons by FISH revealed significant variation from one tumor to another in the level of copy number increase (from one extra copy to more than 40 copies/cell), the size of the region affected (from 10–20 Mb to regions limited to one of the probes tested), and the number of regions involved in copy number increase at 20q13 (1–2 separate sites per tumor). This suggests that

while increased copy number of a single small region was common to the majority of the tumors, the genetic mechanisms as well as selection forces causing the increased copy number may be complex. Low-level copy number increases of large regions at 20q13 were often found, sometimes surrounding the narrow regions of high-level copy number. This low-level gain may represent an early event in the step-wise selection of high copy numbers levels. The common finding of large regions of increased copy number and the occasional presence of distinct sites of high-level copy number increase outside the critical region, suggest that more than one gene in the 20q13 region may be affected.

The association of poor prognosis with increased copy number at 20q13 detected using CGH suggests the importance of the general region. However, the clinical significance of increased copy number at or near RMC20C001 in breast cancer progression is still unclear. Now that the region has been defined precisely, it will be possible to study prognostic associations in large series of breast cancers.

All the currently reported cloned genes in the 20q13 region were excluded as candidate oncogenes based on their chromosomal location and/or absence of high-level copy number increase in breast cancer cell lines and primary tumors. Therefore, it is likely that the gene (or genes) that are selected for by this copy number increase are currently unknown. The RMC20C001 cosmid probe defining the common region of high copy number increase provides a starting point for positional cloning of a gene that may play an important role in the development of certain aggressive forms of breast carcinomas.

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